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Extraction of Rennet From Fresh Frozen Vells

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EXTRACTION OF RENNET FROM FRESH FROZEN VELLS

by

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of

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in

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1968

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Neil Harvey Clarke

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Extraction of Rennet from

Fresh Frozen Vells

by

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Utah State University, 1968

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Department: Food Science and Technology

A method was developed for the extraction of rennet from fresh frozen vells. Frozen vells were partially thawed and minced in a Hobart "Wonder worker." The tissue was treated with 2.0 per cent potassium alum and allowed to stand for 20 hours before sufficient 0.45 M disodium phosphate was added to bring the pH of the mixture to 5.7. Dry sodium chloride was added until the salt concentration in the moisture of the mixture was 10 per cent. The tissue was then mixed with 3/4-inch pumice gravel in a volume ratio of 1 to 2 and packed into extractor columns. The tissue was extracted continuously with a 10 per cent sodium chloride solution. The yield of enzyme and extract viscosity from 60 undried vells was compared with that from 60 dried vells. The undried vells yielded 2.99×10^6 R.U. in 86 liters of extract and the dried vells yielded 3.03×10^6 R.U. in 74 liters of extract. The viscosities of the initial extracts were 5.8 and 6.2 centipoise respectively.

Drying temperature, moisture content and pH had no effect on extract viscosity. Viscosities of extracts were

reduced by aging the dried vells prior to extraction, adjusting sodium chloride to 10 per cent in extracting solutions, and adding 2 to 3 per cent potassium alum to undried vell tissue and holding for 20 hours before neutralizing to pH 5.7 $\pm$ 0.2 with disodium phosphate.

The mean activity value of extracts representing 96,000 vells was 45.5 R.U. with a standard deviation of $\pm$ 19.57 for individual vells. This variation in enzyme content precluded useful comparisons of yield based on small numbers of individual whole vells.

(55 pages)

INTRODUCTION

Rennin is extracted from the mucosa of the abomasum of veal calves (vells), and the extract used to coagulate milk for cheese making. In Europe, New Zealand, and Australia, stomachs are inflated and dried to obtain dry-blown vells; while in the United States, flat-salted vells are prepared by opening the stomachs and packing them with large quantities of dry sodium chloride.

Extraction is hampered by the presence of slimy mucins which form part of the lining of the stomach. It is necessary to store dry-blown vells for up to one year before the slimy properties of the mucous substances are reduced to the point where extraction is feasible. This problem is not experienced to the same degree with flat-salted vells. It appears that drying vell tissue after it has been saturated with sodium chloride causes some alteration of the mucins without affecting enzyme activity. Except for the problem with mucins, there are considerable advantages to extracting dry-blown rather than flat-salted vells. They are easier to handle and store, and they fit better into continuous extraction systems.

During continuous extraction of flat-salted vells the concentration of rennin builds up more slowly than that of sodium chloride. When the salt concentration approaches 25 per cent the rennin is salted out of solution, leaving an extract high in sodium chloride and low in enzyme concentration. With dry-blown

vells an extract of constant sodium chloride concentration and high enzyme activity can be obtained.

The object of this study was to examine the properties and characteristics of slime-forming mucins in vell tissue and to study ways of altering them to permit extraction of fresh vells. If successful this would eliminate the long storage periods and the physical loss of enzyme-containing tissue that occurs during preparation of dry-blown vells at slaughter houses.

REVIEW OF LITERATURE

Mucins and their functions

Mucins are described as viscous, soluble, biological substances consisting mainly of mucopolysaccharides and muco-proteins. Mucopolysaccharides are polysaccharide-protein complexes with carbohydrates as their major constituents. Mucoproteins are composed of the same substances but with proteins as major components (6).

The main function of mucins is to lubricate moving body parts. In the mammalian body mucins are associated with many functions, particularly anatomical and digestive movements. The efficiency of these biological lubricants depends on their high viscosity and their ability to form structural gels at low concentrations. These lubricants have an extra-cellular environment and show a marked degree of resistance to non-specific enzymes. Gastric mucins, in lubricating the passage of digesting foodstuffs in the intestine, are required to resist powerful intestinal enzymes if their action is to continue (6).

Possibly owing to their extracellular distribution, mucins characteristically appear to be in a steady state involving simultaneous biosynthesis and degradation (6).

State of hydration of mucins

Dehydration of mucins with desiccants or hydrophilic solvents modifies their solubility. The mucins are associated

structurally with extensive water shells, removal of which causes collapse of the macromolecular structure. The concept of bound water has been introduced to denote a minimum degree of hydration essential to maintain this structure (6).

Rigorous drying causes introduction of lactone bridges into acid mucosubstances; and in some compounds which contain nucleic acids, drying causes hydrolysis of labile linkages (5).

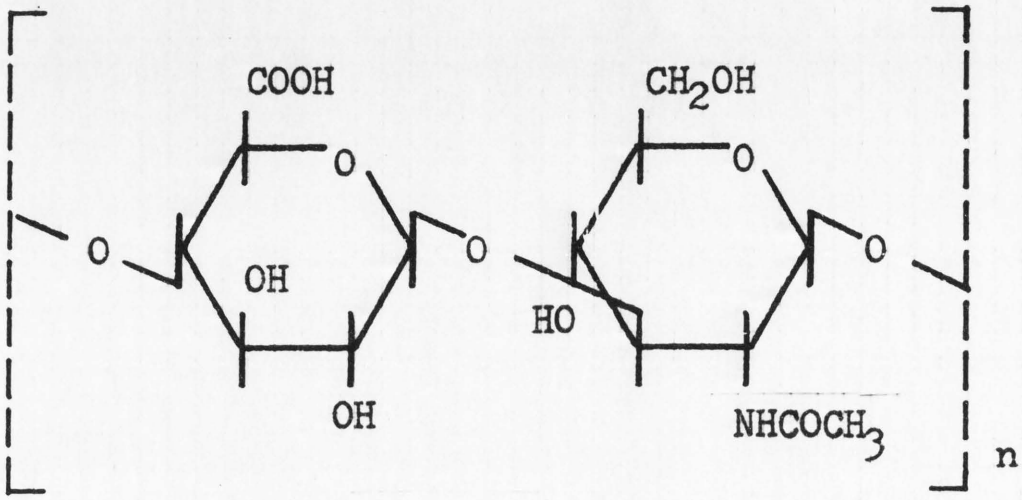
Components of mucins

Four complex polysaccharides can be separated from barium salts of hog gastric mucosa by ethanol fractionation (6). These are heparin, two chondroitin sulfates (21) with specific optical rotations in water of -52° and -28° respectively, and a neutral mucosubstance with blood group specificity (blood group substance). Other components have been reported (7, 11), but some workers (22) are of the opinion that these are various sulfation levels of the same polysaccharides. Hyaluronic acid is also present in many of the polysaccharides of gastric mucins. It consists of repeating equimolar units of acetyl glucosamine and glucuronic acid with alternating β 1,4 and β 1,3 linkages, as shown in Figure 1 (24). It is extracted from tissue with water and forms extremely viscous solutions if it has been prepared without the use of alkali or not precipitated by glacial acetic acid. Chondroitin sulfates may be extracted with disodium phosphate solutions, strong sodium chloride solutions or alkali, while heparin is extracted with alkali after tissue autolysis (18).

Heparins are the most highly ionized polymeric substances

Figure 1. Repeating unit of hyaluronic acid (24)

Figure 2. Postulated repeating unit of heparin (24)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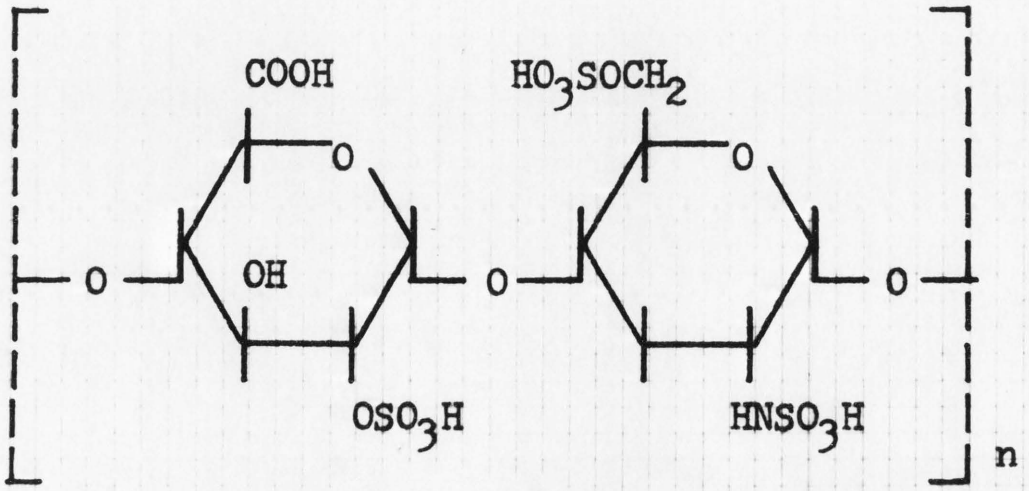
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O
O
O
O
OH
O
OSO3H
HNSO3H
n

in the animal body. Analysis of purified heparin, from lung and liver tissue, has shown D-glucosamine and D-glucuronic acid to be the principal monosaccharide constituents (25). Kent (6) suggested that the amino groups are partly free and partly sulfated but that the number of sulfate groups varies considerably. He also reported that all the sulfur is present as ester sulfate, five such esters being distributed in every four monosaccharide residues. Other workers (24) indicate that there are six sulfate esters present in every four monosaccharide residues but are not certain whether all or only a portion of the glucuronic acid residues carry an ester sulfate group. Their postulated repeating unit or heparin is presented in Figure 2 (24). Heparin has the ability to enter into physico-chemical combinations with certain serum components and probably for this reason is also a powerful inhibitor of a variety of enzymes (6). It also interferes with the coagulation of blood (18).

Chondroitin sulfate contains equimolar parts of acetyl galactosamine, ester sulfate, and glucuronic acid (10). Enzymatic studies have shown the occurrence of at least three forms of chondroitin sulfate that have been designated A, B, and C, and which are distributed in varying amounts in different tissues (13). Chondroitin sulfates A and C differ only in the position in which the sulfate is esterified to the galactosamine moiety. Both of these contain alternating β 1,3 and β 1,4 linkages. The polymer that was termed chondroitin sulfate B yields L-iduronic acid on hydrolysis and not D-glucuronic acid, the two differing only in configuration at the C-5 carbon atom. It is named dermatan

sulfate and contains alternating α 1,3 and β 1,4 linkages. Repeating units of these three polymers are shown in Figure 3 (24).

Viscous properties of chondroitin sulfates from cartilage correspond to the behavior of an extended polyelectrolyte in a highly ionized condition. The viscosity is decreased by salts to an extent dependent on the size and concentration of the added cations. Calcium chloride is more effective than sodium sulfate or sodium chloride. It appears that the viscosity contribution of the polyion in the presence of added salt depends only on the counterion charge and concentration and not on the ionic strength. This may be due to the high negative potential of the chondroitin sulfate polyion and its immediate electrical environment which consists almost entirely of positive ions. Negative ions are therefore unable to appreciably influence the properties of chondroitin sulfate solutions (12).

Both $Co(NH_3)_6Cl_3$ and $CaCl_2$ produce a considerably tighter polymer coil and lower viscosity than is found at high NaCl concentrations. This may be an effect due to electrostatic interaction of a polycation with more than one anionic site which is in addition to contraction of the coil caused by a simple reduction of the net charge (12).

The mechanical properties and degree of hydration of chondroitin sulfate and its environmental electrolytes are related and regulated by conditions of salt concentration and pH (17).

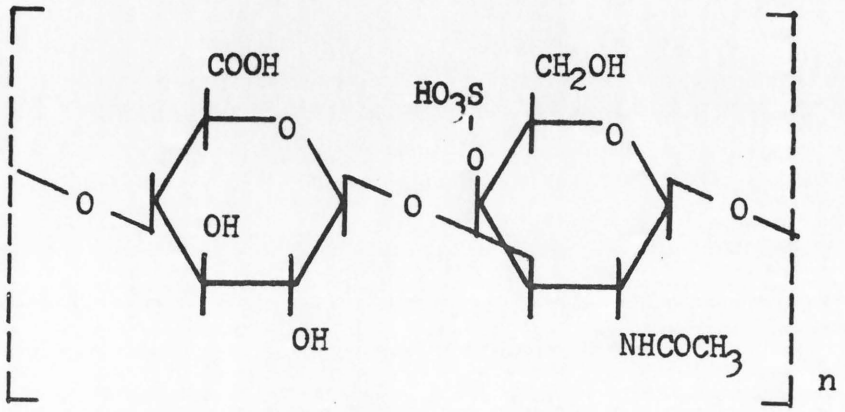
Blood group substances are present in a variety of mammalian secretions as well as erythrocytes, e.g. hog

Figure 3. Repeating units of polymers in the chondroitin sulfate group (24)

A. Chondroitin sulfate A

B. Chondroitin sulfate C

C. Dermatan sulfate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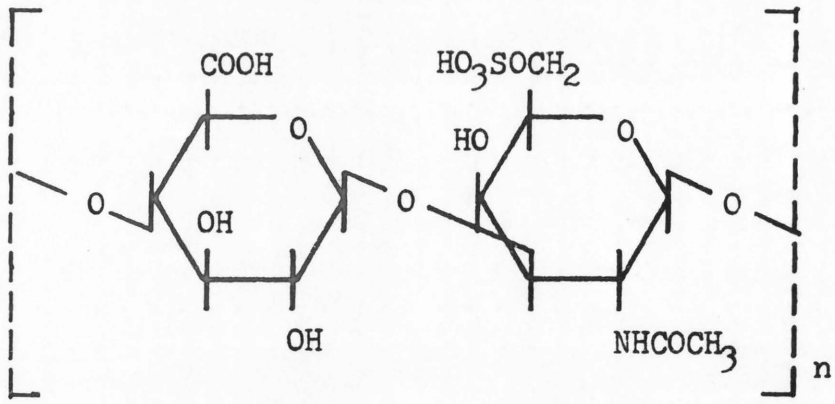
CH2OH
O
O
O
O
O
O
OH
OH
NHCOCH3
n

A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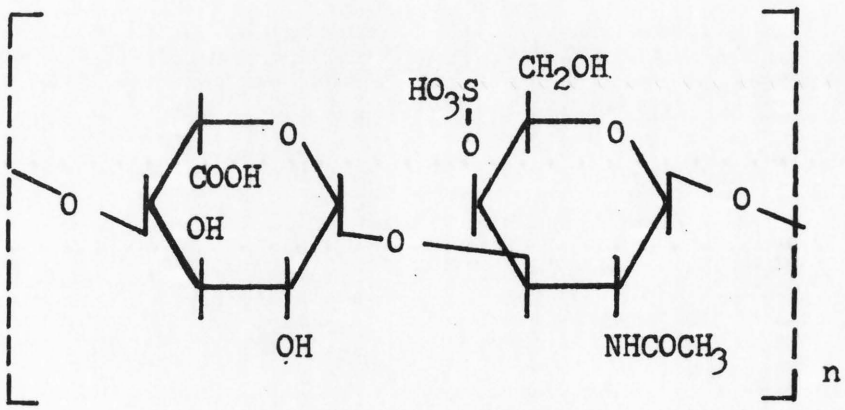
HO
O
O
O
O
O
OH
OH
NHCOCH3
n

B


HO3S
CH2OH
O
O
O
O
O
COOH
OH
OH
NHCOCH3
n

C

gastric mucins, horse saliva, spermatozoa, etc. (6). Both purified A and O substances of hog gastric mucin are mucopolysaccharides of high molecular weight and are composed of carbohydrates and peptides. Removal of the peptide constituent is accompanied by loss of specificity (14). Acid hydrolysis of blood group substances liberates L-fucose, D-glucosamine, D-galactosamine, D-galactose, and about eleven amino acids, mainly methionine and alanine (6).

Partially purified blood group substances retained the viscosity of the original mucin extract after being separated from autolyzed mixtures by ethanol (20) or 90 per cent phenol (14).

Rennet extraction

A continuous rennet extraction process has been described for flat-salted vells (19). Vells were opened at the slaughter house and packed in wooden barrels with large amounts of dry sodium chloride. At the extraction plant solid salt was removed by washing the vells in saturated sodium chloride in a rotating perforated cylinder. The vells were stretched, defatted, and hung on dowels in an air circulating oven at 43.3 C for 20 hours. Dried vells were ground in a hammer mill with excelsior or glass wool in the ratio of one to three. The excelsior or glass wool acted as an inert support without which the slimy mucins kept the extracting fluid from percolating. Moreover, mucins made the extracting mass so thick that it could not be stirred or centrifuged (19).

The ground mixture from the hammer mill was conveyed into

2,000-gallon wooden vats, each holding the equivalent of 10,000 to 15,000 vells. A 10 per cent solution of sodium chloride at pH 5.95 to 6.05 was used to cover the vat contents. The liquid at 2 to 5 C was circulated for two days with countercurrent flow (19).

The extract, which contained considerable prorennin, was activated by adjusting the solution to pH 4.6 with 18 per cent hydrochloric acid. After 14 to 30 hours, when there was no further increase in activity, the pH was raised to about 5.7 with a water slurry of sodium bicarbonate. Preservatives, coloring matter, etc. were added; and the rennet filtered in two stages prior to final adjustment of enzyme activity (19).

Dry-blown vells are prepared by expelling the stomach contents and tying a string around one end of the stomach which is then infalted with air. The other end is tied off and the stomach hung overnight in a circulating air oven at 37 to 49 C. The strings are then cut off and the vells packed in bundles for shipment to the processor (15).

The extraction process is similar to that described for flat-salted vells. Inert gravel may be used as a support medium in which case the gravel and tissue are mixed after the tissue is ground.

Alum treatment of rennet extracts

Potassium alum has been used to clarify rennet extracts in the preparation of crystalline rennin by forming "in situ" precipitates of aluminum phosphate (2) and aluminum hydroxide

(2, 3). These precipitates absorb non-rennin impurities, including mucins, very strongly and are easily removed by filtering (2) or centrifuging (3). The soluble rennin, thus freed from the mucous impurities, can be crystallized. The use of NaOH for neutralizing potassium alum causes loss of some enzyme activity (2, 3), but Na_2HPO_4 can be used without causing loss of activity (2).

Van der Burg and Van der Scheer (23) used a similar method to obtain a clear solution of rennet extract. A flocculent precipitate was formed within the liquid by successive additions of potassium alum and disodium phosphate solutions. A pH of 4.7 to 5.0 was obtained by adding 0.2 per cent alum to activate the extract. Once the extract was fully activated 0.6 to 0.7 per cent additional alum was dissolved in the solution. Sufficient disodium phosphate was added to precipitate all the aluminum and raise the pH to 5.3 to 6.3. Usually 1.6 to 1.8 per cent disodium phosphate was required, and this was added as a 10 per cent solution. After standing for one day during which the precipitated aluminum phosphate settled to the bottom of the tank, clear rennet was siphoned off and the rest mixed with filter aid and filtered. This procedure resulted in an insignificant loss of enzyme activity (23).

Variability of vells

Individual vells vary widely in enzyme content, depending on the diet of the individual animals and on their age at the time of slaughter (19). Leitch (9) reported that the enzymatic secretion of the very young calf is predominantly rennin, but

at the age of five months, the calf's stomach yields pepsin almost to the exclusion of rennin.

Placek (19) claimed that evaluation of 10 to 20 vells from a batch of 700 gave a reliable estimate of the enzyme content for the batch. Legg (8) found that samples of dry-blown vell tissue taken after grinding did not predict accurately the enzyme content of the batch. Samples of ground vell tissue were collected mechanically as the vells were ground and also by hand after grinding was complete. Each sample, equivalent to 20 out of 1,750 vells, was extracted and the yield of the batch estimated. This estimate was compared with the actual yield obtained from commercial extraction for each batch. Samples collected mechanically varied in enzyme activity by as much as 24 per cent from the commercial extraction, while samples collected by hand varied up to 14.1 per cent. A total of 12 comparisons were made by mechanical sampling and 11 by hand sampling (8).

Over a two-year period samples of five vells were taken at random from lots of 1,000 dry-blown vells from a single slaughter house. In all, 96,000 vells were sampled. Each five-vell sample was cut into strips and extracted with 1.2 gallons of 6.0 per cent sodium chloride for five days at 4.4 C. The extracts, which were tested, varied from 30.0 to 74.4 rennin units per ml (8).

Small samples of vells from the same population showed a large variation in enzyme content (8). Therefore it is impossible to compare the enzyme content from small samples of vells after different treatments. For this reason samples were taken from

large amounts of ground vell tissue that had been well mixed. This was considered to give representative samples of tissue containing approximately equal quantities of enzyme. Only by this method was Legg (8) able to compare the effect of vell treatments on enzyme yield.

METHODS

Preparation of vell tissue

Vell tissue consisted of the stomachs of two- to five-day-old, milk-fed calves. Stomachs were opened to remove the contents, packed in Polythene-lined cartons, and frozen. Known numbers of vells were weighed, partially thawed, and ground in a Hobart electric cheese grinder or a Hobart "Wonder worker." The ground material was refrozen until required.

Drying vell tissue

Thawed, ground vell tissue was spread in a thin layer (1/8 inch) on parchment paper and dried at 25 $\pm$ 1 C in a current of air from a small electric fan for 16 to 20 hours. The parchment paper and tissue were minced in a hand grinder to reduce particle size and, when necessary, further dried in a desicator over concentrated sulfuric acid for one to three days.

Alum treatment of vell tissue

Varying quantities of 0.16 M potassium alum solution were mixed into vell tissue and left for 10 minutes to 24 hours before an equal volume of 0.4 M disodium phosphate solution was added. Dry sodium chloride was added to give a concentration of 10 per cent throughout the liquid phase of the system. Solutions of potassium alum ($AlK(SO_4)_2.12H_2O$) and disodium phosphate (Na_2HPO_4) were prepared at concentrations of 0.16 M and 0.4 M respectively.

This permitted equal volumes of these two solutions to give a precipitated mixture of pH 5.7 ± 0.1.

Enzyme extraction

Vell tissue, prepared by one of the above methods was extracted by a 10 per cent sodium chloride solution at pH 5.7 ± 0.2. Sodium benzoate (0.75 per cent) was added as a preservative. Extraction was carried out by a batch as well as a continuous process. During batch extraction 150 g of wet tissue containing approximately 82 per cent moisture was extracted with 250 ml of 15.2 per cent sodium chloride, while 23 g of dry tissue was extracted with 380 ml of 10 per cent sodium chloride. Each batch was extracted for five days at 4 C, and was agitated daily. The extract was separated from the tissue by filtering through a coarse bed of excelsior.

Continuous extraction was carried out on wet and dry minced vell tissue, prepared as for batch extraction but mixed with an inert support material of 3/4-inch pumice gravel. The tissue-gravel mixture was packed into extractor columns in 12-inch layers between 2- to 3-inch layers of gravel. There were five columns, each 5 inches in diameter and five feet tall. The continuous extraction apparatus is illustrated in Figure 4. Each column was loaded with tissue equivalent to 20 to 25 vells and approximately 3 gallons of gravel. Columns were linked in series with tubing so that brine flowed by gravity from the top of one column into the bottom of the next. Flow rates were varied from 100 to 600 ml per hour by adjusting the length of stroke on a CRC

Figure 4. Apparatus used for continuous enzyme extraction

Vibrostaltic pump. Brine was delivered from the pump into a holding vessel above the level of the top of the columns and gravitated into the bottom of the first column. Countercurrent flow was employed in extraction so that fresh brine was fed into the most exhausted column and the final extract taken from the top of the last column loaded. This procedure allowed a maximum quantity of brine to wash the tissue that had been partially extracted and still keep the final extract high in enzyme activity. Columns were extracted for four to six days before discarding the spent contents.

Viscosity measurements

Viscosity of rennet extracts was measured by timing the flow of liquid between two arbitrary marks on a 10-ml pipette which had the tip orifice enlarged. Readings were taken at 4 C immediately after the liquid was separated from the tissue. Readings were expressed relative to 10 per cent sodium chloride solutions, and densities were calculated by weighing 50 ml of each extract. Relative viscosities were calculated from the following formula:

$$\eta_R = \frac{\text{Flow time}_{(Sa)} \times \text{Density}_{(Sa)}}{\text{Flow time}_{(St)} \times \text{Density}_{(St)}}$$

η_R = Viscosity of extracts relative to 10 per cent sodium chloride

(Sa) = Sample

(St) = Standard of 10 per cent sodium chloride

The viscosity of 10 per cent sodium chloride at 4 C was measured with a Brookfield LV viscometer. Absolute viscosities of the extracts were calculated from the formula:

$\eta = \eta_R \times 4.0$

η = Absolute viscosity, centipoise.

4.0 = Viscosity in centipoise of 10 per cent sodium chloride at 4 C.

Measurement of enzyme activity

The milk clotting test of Ernstrom (4) was utilized in which substrates were always prepared from a constant supply of nonfat dry milk. A standard-strength rennet extract* containing an arbitrary 100 rennin units per ml was used as a reference for every test.

Mucin solution

An extract was prepared as described in the batch extraction method above, except 400 ml of brine was used. The extract was dialyzed against distilled water containing 1.0 per cent sodium benzoate at 4 C to remove the sodium chloride. When the water was free of chloride ions, as indicated by the silver nitrate test, the solution was stored at 4 C until required.

Measurement of aluminum binding by vell tissue

Binding of potassium alum by fresh vell tissue and mucins

*Supplied by Dairyland Food Laboratories, Waukesha, Wisconsin.

was determined by dialysis equilibrium in which the excess aluminum in tissue suspensions was measured after dialysis against solutions of known potassium alum concentration. Weighed samples of tissue and mucins were placed in dialysis tubing and suspended in a solution containing a known concentration of alum. After five days at 4 C, the materials inside and outside the dialysis casings were reweighed and analyzed for aluminum and moisture. Aluminum was expressed as milligrams of aluminum per milliliter of water in tissue or mucins. This was compared with the milligrams of aluminum per milliliter water in the solution outside the dialysis casing. A higher concentration of aluminum in the water inside the dialysis casing was indicative of aluminum being bound by tissue or mucins. This in turn was used to calculate bound aluminum per gram of tissue or mucins.

The absorption of aluminum with time was followed by adding 20 ml of 0.16 M potassium alum solution to 40 g of vell tissue and allowing it to stand for varying times before decanting the excess liquid. The tissue was then analyzed for aluminum.

Aluminum analysis

Samples were analyzed for aluminum by an official AOAC method (1). Five-ml samples of solution or 5 ml of solution obtained by heating 10 g of tissue with 5 ml of concentrated hydrochloric acid plus 25 ml water were used in duplicate assays.

Moisture analysis

The moisture content of samples for the above tests was determined by heating 2- to 4-g samples in 2-inch diameter

containers to 102 C in a forced draft oven for 16 hours (1).

Statistical analysis

The standard deviation of enzyme activities from the mean of 96 extracts representing five vells each was calculated by the machine formula from Ostle (16). This value was multiplied by $\sqrt{5}$ to give the standard deviation of enzyme activity for individual vells (16).

RESULTS

Effect of vell drying temperature on extract viscosity

Two portions of thawed, ground vell tissue were dried under a vacuum of 24 ± 1 inches of mercury to 7.2 and 6.5 per cent moisture at temperatures of 51 ± 2 and 61 ± 2 C respectively. Each portion was batch extracted and the extract viscosities found to be 5.3 and 5.1 centipoise.

The small difference between viscosities indicated that drying vells within the range of 51 to 61 C did not appreciably affect the viscosity of their extracts.

Effect of moisture in dried vells on extract viscosity

Representative portions of ground vell tissue were dried at 25 ± 1 C to moisture levels ranging from 3.0 to 13.0 per cent. Each portion was batch extracted and the viscosity of each extract measured. The results are presented in Table 1 and show that viscosities ranged from 5.8 to 6.4 centipoise.

These data indicate that within the range of 3.0 to 13.0 per cent moisture there was no relationship between the viscosity of the extract and the moisture content of the vell tissue.

Table 1. Effect of moisture content in dried vells on extract viscosity

Lot number	Moisture in tissue (%)	Viscosity of extracts (cps)[a]
1	9.5	6.0
2	3.0	6.4
3	4.4	6.0
4	13.0	5.8

[a]centipoise

Effect of age of dried vells on extract viscosity

A large amount of ground vell tissue was dried at 25 ± 1 C to 8.5 per cent moisture and batch extracted after being held at 4 C for varying periods of time. Average viscosities for five replicate extracts at each age are presented in Table 2. Extract viscosities were 6.7 and 6.1 centipoise from tissue that had been dry for 18 and 33 days respectively.

Table 2. Effect of age of dried vell tissue on extract viscosity

Age of dry tissue (days)	Viscosity of extracts (cps)[a]	Standard deviation
18	6.7	± 0.15
19	6.6	± 0.28
33	6.1	± 0.24

[a]centipoise

These data suggest that a relationship exists between extract viscosity and the length of time vell tissue had been dry. This observation agrees with what has been found in practice. Vells that have been dry for several months produce less viscous extracts than freshly dried vells.

Effect of pH on extract viscosity

Five 23-g batches of dried vell tissue were mixed with 380 ml of 10 per cent sodium chloride solution. The slurries were adjusted to pH 4.8, 5.0, 5.2, 5.5, and 5.7 respectively with 10 per cent hydrochloric acid. These pH values increased 0.3 to 0.6 pH units after five days to give values in the desired pH range of 5.4 to 6.0. The viscosity of each extract was measured after it had been separated from the tissue. The results are presented in Table 3.

It appears that the pH of rennet extracts within the range of 5.4 to 6.0 had no effect on their viscosities.

Table 3. Effect of pH on extract viscosity

pH of extract		Extract viscosity (cps)[a]
Initial	Final	
4.80	5.40	6.7
5.00	5.55	6.5
5.20	5.65	6.7
5.50	5.85	6.5
5.70	6.00	6.5

[a] centipoise

Effect of sodium chloride concentration on extract viscosity

Sodium chloride concentrations ranging from 6.0 to 14.0 per cent were used in batch extractions of veal tissue dried at 25 ± 1 C and containing 8.5 per cent moisture. Extract viscosities varied from 6.0 to 6.9 centipoise. Results are presented in Table 4.

Table 4. Effect of sodium chloride concentration on extract viscosity

Sodium chloride concentration (%)	Extract viscosity (cps)[a]
6.0	6.9
8.0	6.4
10.0	6.3
12.0	6.0
14.0	6.1

[a] centipoise

A small decrease in extract viscosity with increasing sodium chloride concentrations was evident, but the difference between 6.0 centipoise at 12.0 per cent sodium chloride and 6.3 centipoise at 10.0 per cent salt was not considered of sufficient significance to alter the concentration of ensuing brines in later experiments. A sodium chloride concentration of 10 per cent was originally selected for extractions as this is used in some commercial rennet extractions (19). The above data show that an extract containing 12.0 per cent sodium chloride had a slightly lower viscosity than one containing 10.0 per cent

salt, but within this range of viscosities the difference was considered to be of no practical significance.

Effect of $KAl(SO_4)_2$ concentrations on extract viscosity

Preliminary trials showed that an extract of low viscosity could be obtained by treating fresh frozen vell tissue successively with potassium alum and disodium phosphate solutions.

A control extract was obtained by batch extracting two 150-g lots of untreated, ground vell tissue.

Batch extracts were obtained from wet vell tissue previously treated with 0.68 to 3.40 per cent potassium alum. The mixture was neutralized two hours later with 0.4 M disodium phosphate to a pH of 5.7 $\pm$ 0.2. The extract was decanted and the viscosity measured. Results presented in Table 5 show that viscosities varied from 10.3 to 35.1 centipoise.

These data showed that rennet could be readily extracted from vells treated with 2 to 3 per cent alum.

Table 5. Effect of alum concentration on extract viscosity

Alum concentration (%)	Extract viscosity (cps)[a]
0.00	105.6
0.68	35.1
1.36	16.2
2.04	11.2
2.72	10.3
3.40	11.8

[a] centipoise

Effect of reaction time of $KAl(SO_4)_2$ in tissue on viscosity and activity of rennet extract

Potassium alum (0.16 M) was mixed into six 150-g lots of ground veil tissue to concentrations of 2.04 per cent. At various intervals 0.4 M disoidum phosphate solution was added to neutralize the mixtures to pH 5.7 ± 0.2. Each lot was batch extracted with 250 ml of 15.2 per cent sodium chloride which remained in contact with the tissue for five days. The viscosity and enzyme activity of each extract was determined and the results presented in Table 6. Viscosities ranged from 9.0 to 25.8 centipoise, but enzyme activity was reasonably constant between 104 and 112 rennin units per ml.

Table 6. Effect of reaction time of 2.04 per cent alum in tissue on viscosity and activity of rennet extract

Time before neutralization (hr)	Extract viscosity (cps)[a]	Enzyme activity (R.U./ml)
20.0	9.0	108
4.0	10.3	112
2.0	12.7	111
1.0	14.2	104
0.5	11.4	107
0.25	25.8	107

[a] centipoise

Increasing exposure of veil tissue to potassium alum before neutralization with disodium phosphate caused no change in rennin activity but produced a sharp decrease in extract

viscosity during the first hour followed by a moderate decrease up to 20 hours.

Binding of potassium alum by vell tissue and mucins

Samples of ground vell tissue (47.7 g) and mucin solution (75.8 g) containing 84.5 and 99.2 per cent moisture respectively were weighed into separate dialysis casings. The mucin solution was prepared as described in the methods section. Each casing was immersed for three days in a beaker containing 250 ml of 0.16 M potassium alum solution at 4 C and allowed to approach equilibrium. Casings were removed from their dialyzing solutions and their contents weighed and analyzed for aluminum. The concentrations of aluminum per milliliter of water present in the tissue and mucin solutions were calculated and compared with the final aluminum concentration in the dialyzing solution. At equilibrium the tissue and mucin solution contained 24.3 and 13.0 per cent more aluminum per milliliter of water respectively than their corresponding dialyzing solutions. It was assumed that the excess aluminum was bound by the tissue or mucins. Results are presented in Table 7 and show that vell tissue bound 25.9 mg aluminum per gram of solids. Mucins, on the other hand, bound 327.4 mg aluminum per gram of solids or more than ten times the amount bound by vell tissue.

Rate of aluminum binding by vell tissue

Ground vell tissue was divided into eight 40-g lots and each was thoroughly mixed with 20 ml of 0.16 M potassium alum

Table 7. Aluminum binding by vell tissue and mucins as determined by equilibrium dialysis

	Initial conditions				Equilibrium conditions					
	Weight (g)	Moisture (%)	Volume (ml)	Al conc. (mg/ml)	Weight (g)	Moisture (%)	Al conc. (mg/ml)	Volume (ml)	Al conc. (mg/ml)	Al conc. (mg/g)
Vell tissue	47.7	84.5			49.7	86.2	4.46			25.9
Dialyzing solution			250	4.22				250	3.59	
Vell mucin	75.8	99.2			58.5	96.2	3.55			327.4
Dialyzing solution			250	4.22				272	3.09	

solution (2.0 per cent alum w/w). The free liquid was drained from the samples after standing for various periods up to 20 hours. After draining, each sample was analyzed for aluminum and moisture. The results presented in Table 8 show an increase in amount of aluminum bound from 3.48 mg/g of dry tissue after 15 minutes to 10.71 mg/g after 8 hours. That this upward trend continued is shown in Table 7 where after 72 hours, vell tissue had bound 25.9 mg aluminum per gram dry tissue.

Table 8. Rate of aluminum binding by vell tissue

Time before draining (hr)	After draining	
	Moisture in tissue (%)	Aluminum bound (mg/g)
0.0	81.8	0.00
0.25	83.4	3.48
0.50	83.0	5.42
1.0	83.6	4.72
2.0	81.7	6.42
4.0	83.4	7.01
8.0	83.7	10.71
20.0	84.9	9.54

If no aluminum had been bound by the vell tissue, one would have expected to find 7.17 mg aluminum per gram of dry tissue after equilibration as the aluminum would have been evenly dispersed in the water phase. The results in Table 8 show that this value was exceeded and produced further evidence that aluminum was actually bound by vell tissue.

Variability among small samples of vells

Data supplied by Legg (8) were analyzed to show the variability in enzyme content between small samples of vells. A total of 96,000 dry-blown vells from one slaughter house were sampled over a two-year period by randomly selecting 5 vells from each batch of 1,000. Each sample of 5 vells was cut into strips and extracted with 1.2 gallons of 6.0 per cent sodium chloride solution for five days at 4.4 C. Extracts were assayed for enzyme content and the results presented in Figure 5.

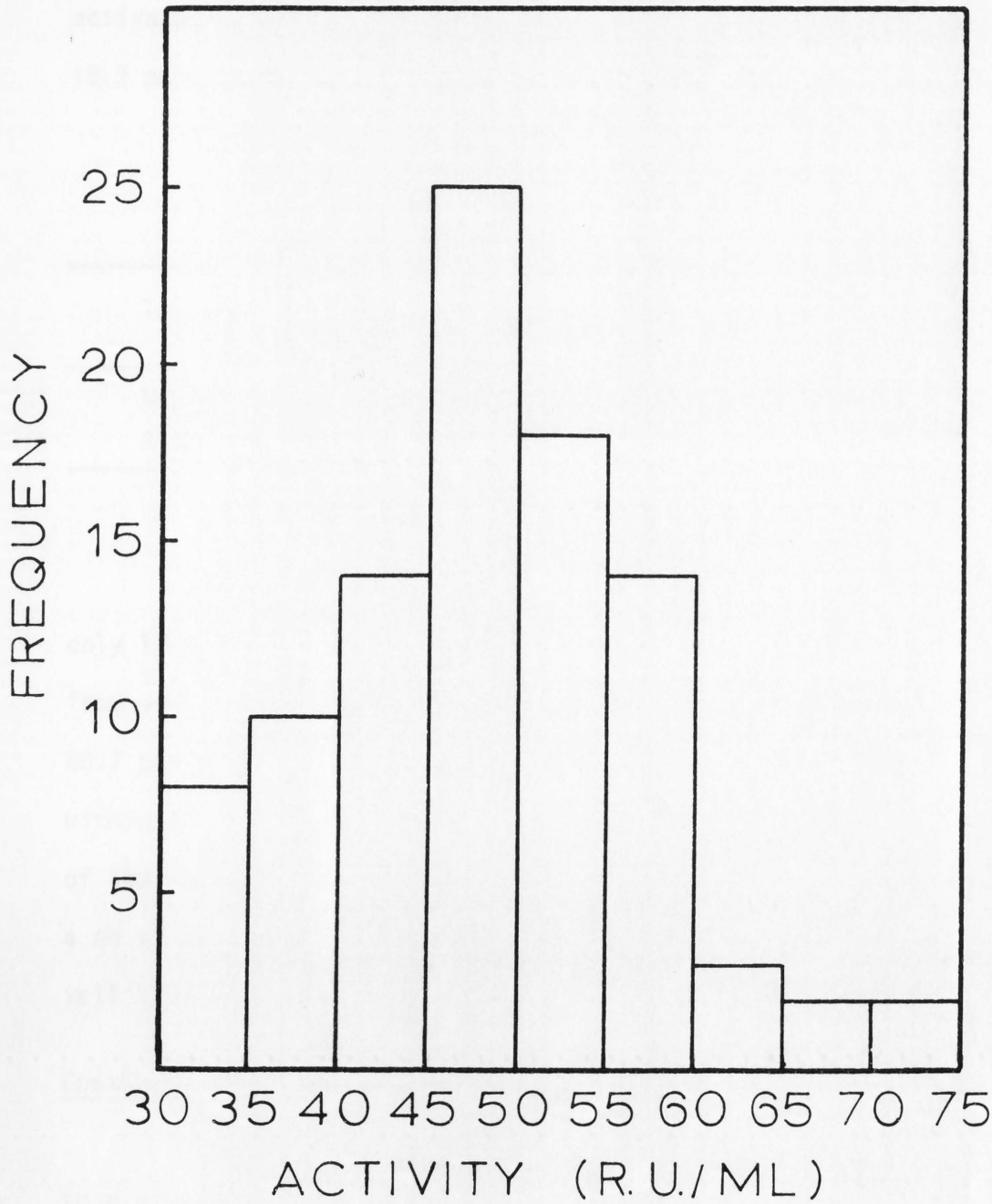
The average activity for the 96 samples was 45.50 R.U./ml with standard deviations of $\pm$ 8.70 for samples of 5 vells and $\pm$ 19.57 for individual vells.

These results emphasize the extensive variation in the rennin content of individual calf stomachs and the hopelessness of trying to compare yields by sampling small numbers of whole vells and assuming that they are representative.

Activation of prorennin by potassium alum treatment of vells

A large quantity of ground vell tissue was divided into two representative portions. One was dried at 25 $\pm$ 1 C while the other was treated for 20 hours with 2.0 per cent potassium alum (added as a 0.13 M solution) before neutralization to pH 5.7 $\pm$ 0.2 with 0.47 M disodium phosphate. Extracts of both portions were obtained by continuous extraction and were assayed both before and after activation. Assays following activation measured the total rennin content of the extract while tests on

Figure 5. Distribution of enzyme activity among 96 extracts, each one representing 5 vells selected at random from 1,000 vells


FREQUENCY
25
20
15
10
5
30 35 40 45 50 55 60 65 70 75
ACTIVITY (R.U./ML)

unactivated samples measured only the active rennin and not the unactivated prorennin. Results presented in Table 9 show that alum-treated vells produced extracts containing 88.7 per cent activated rennin while extracts of dried vells contained only 18.9 per cent.

Table 9. Activation of prorennin by potassium alum treatment of vells

Treatment	Replicates	Active rennin (%)	Standard deviation
Drying	2	18.9	± 2.9
Alum	3	88.7	± 4.1

These data show that extracts from dried vells contained only 18.9 per cent of the potential activity whereas extracts from vells treated with 2.0 per cent potassium alum contained 88.7 per cent of the enzyme in its active form. Activation within the tissue no doubt resulted from the acid character of the potassium alum. A 0.16 M solution of potassium alum had a pH of 2.8 ± 0.1, and addition of 2.0 per cent potassium alum to vell tissue reduced the pH of the tissue from 6.1 to 3.6.

Continuous extraction

A total of 120 fresh frozen vells were weighed, ground in a Hobart "Wonder worker" and divided into two representative portions equivalent to 60 vells each. One portion was dried at 25 C while the other was treated with 2.0 per cent potassium

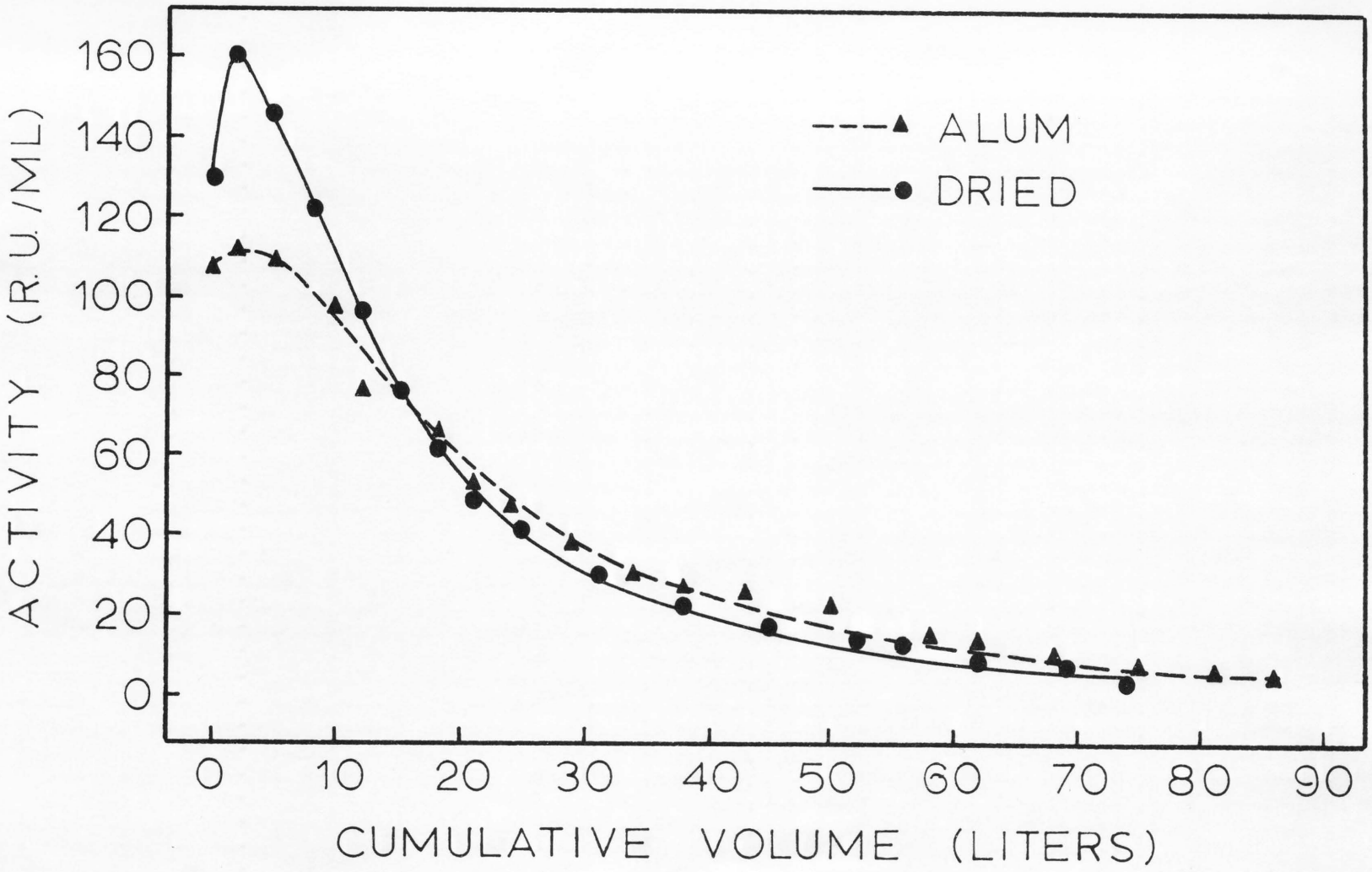
alum added as a 0.13 M solution. After 20 hours the alum-treated tissue was neutralized to pH 5.7 ± 0.2 with 0.45 M disodium phosphate. Enough dry sodium chloride was added to produce a concentration of 10 per cent in the liquid phase of the mixture. Each portion was mixed with 3/4-inch pumice and loaded into three extraction columns.

The first column was filled from the bottom with 10 per cent sodium chloride solution and left 20 hours to allow the enzyme concentration to equilibrate. This liquid was displaced into the second column by fresh brine, and after a further 20 hours the third column was filled in a similar manner. After another 20 hours, continuous flow was begun at 150 to 250 ml/hr. Fractions were collected at intervals from the top of the third column and measured for volume and enzyme activity. Flow was continued through each column until the enzyme activity of the overflowing extract was less than 2.0 R.U./ml, when the spent tissue was discarded.

The enzyme activity and volume of each fraction was measured and plotted in Figure 6. Eighty-six liters of extract were taken from 60 alum treated vells before the enzyme activity was below 5.0 R.U./ml while only 74 liters were required to reduce enzyme activity to the same level from 60 dried vells. These data suggested that rennin is released more rapidly from vells dried at 25°C ± 1 C than from vells treated for 20 hours with 2.0 per cent potassium alum.

A total of 2.99×10^6 rennin units were obtained from

Figure 6. Cumulative volume and enzyme activities of fractions collected during continuous extraction of dried and alum-treated fresh frozen vells


ACTIVITY (R.U./ML)
160
140
120
100
80
60
40
20
0
ALUM
DRIED
0
10
20
30
40
50
60
70
80
90
CUMULATIVE VOLUME (LITERS)

60 alum-treated vells while the same number of dried vells yielded 3.03×10^6 rennin units. The difference between these two values was considered to be within the limits of experimental error. This suggested that potassium alum treatment of vells caused no loss of enzyme as compared with drying vells at 25 C $\pm$ 1 C.

Viscosity of the first 2.4 liters of extract from the dried vells was 6.2 centipoise while the first 1.2 liters of extract from alum-treated vells had a viscosity of 5.8 centipoise. In both cases this was the highest extract viscosity observed from each portion of vells. The difference between these viscosities is considered to be of no practical significance.

It appeared that potassium alum treatment was comparable to drying fresh frozen vells at 25 C $\pm$ 1 C in all aspects except the rate of release of enzyme. This is shown by the higher enzyme activity achieved and the smaller volume of extract required to obtain all the enzyme from the dried vells. This, however, need not be a disadvantage if a countercurrent flow system is used for extraction.

DISCUSSION AND CONCLUSIONS

There is a need in the rennet manufacturing industry for a process whereby rennin can be extracted from fresh vells. Such a process would increase vell handling efficiency by eliminating the need to age dry-blown vells for 9 to 12 months prior to extraction and substantially reduce the cost of rennet manufacture.

Extraction of fresh vells or even freshly dried vells has been limited because of slime-forming mucins that are extracted with rennet to form an extract of high viscosity. These highly hydrated mucins interfere with the release of enzyme from vell tissue and impede the percolation of extracting solutions. This study was an attempt to find ways of reducing the slime-forming characteristics of calf gastric mucins to the point where commercial extraction of freshly dried or even undried vells was feasible.

Extracts representing 96,000 vells had a mean activity value of 45.5 R.U. with a standard deviation of $\pm$ 19.57 for individual vells. To overcome this variation in enzyme content of individual vells large numbers of vells were minced and well mixed before being divided into representative portions for treatment comparisons.

Under the conditions of this study drying temperatures, moisture content, and pH had no obvious effect on the viscosity of rennet extracted from dried vells. It has been suggested

that the solubility of gastric mucins could be affected by drying only when bound water was partially removed (6). Satisfactory reduction in viscosity was accomplished by drying to 13.0 per cent moisture at 25 C $\pm$ 1 C, and no further benefit was achieved by drying vells to lower moisture levels. If the suggested relationship between bound water and mucin solubility is correct, it appeared that a relatively high proportion of the moisture in fresh vell tissue must be in the bound form. When vell drying temperatures were increased from 25 to 61 C there was no significant reduction in the viscosity of rennet extracts. Viscosities were unaffected by pH within the range of 5.4 to 6.0. Trials were limited to this pH range because rennin stability and bacteriological considerations make it desirable to keep rennet extracts within these pH limits.

Factors which affected extract viscosity were age of the dried vells, sodium chloride concentration in the extracting solutions, and concentration and reaction time of potassium alum in undried vell tissue. It was apparent that mucins in dried vells became less soluble during storage. This probably explains the necessity of storing dry-blown vells for several months before they can be extracted under factory conditions. Extract viscosity decreased slightly with increased sodium chloride concentrations between 6.0 and 14.0 per cent. A substantial decrease in viscosity of extracts from fresh alum-treated vells was achieved by adding enough dry sodium chloride to bring the concentration of salt in the moisture of the mixture to 10 per cent.

The addition of 2 to 3 per cent of potassium alum to fresh minced vell tissue followed by a holding period of 20 hours before neutralization to pH 5.7 $\pm$ 0.2 with disodium phosphate permitted easy extraction of rennin with little interference from viscous mucins. It appeared that aluminum ions were capable of binding to the highly ionized mucin molecules, reducing the net charges and enabling the molecules to form tighter coils which produced an extract of lower viscosity. Less than 20-hour exposure of tissue to potassium alum before neutralization reduced extract viscosity to a lesser extent, and longer exposures were not considered necessary for commercial extractions.

Potassium alum treatment of vells also caused activation of most of the prorennin in the tissue due to the low pH of the tissue-alum mixtures. This possibly could be developed to activate all the enzyme prior to extraction and eliminate the necessity of activating the rennet after it is extracted. This would reduce losses of enzyme encountered by activating at pH values less than 5.0 in the presence of large quantities of chloride ions.

The yield of enzyme from the continuous extraction of alum-treated fresh vells was comparable to that obtained from dried vells. Even though it was demonstrated that rennin is more readily released from dried than from fresh tissue, extracts from fresh tissue were still high enough in activity to satisfy the requirements of commercial extraction.

The following procedure for the extraction of fresh

vells is recommended.

1. Vells should be frozen at the slaughter house as soon as practical after removal from the calf.

2. Frozen vells should be partially thawed and minced as fine as possible.

3. Add 2.0 per cent potassium alum, as a solution, to the minced vell tissue and hold at 4 C for 20 hours.

4. Neutralize the alum-tissue mixture to pH 5.7 $\pm$ 0.2 with a 0.4 to 0.5 M solution of disodium phosphate.

5. Add dry sodium chloride to achieve a 10 per cent concentration throughout the moisture in the mixture.

6. Mix support material (3/4-inch inert gravel) with treated vell tissue in ratio of 1 to 2 v/v, and load into extractors.

7. Extract the enzyme, using countercurrent flow, with 10 per cent sodium chloride solution containing 0.75 per cent sodium benzoate at pH 5.7 and 4 C.

LITERATURE CITED

1. Association of Official Agricultural Chemists. 1965. Official methods of analysis. 10th Ed. Association of Official Agricultural Chemists. Washington, D. C. 957 p.

2. Berridge, N. J. 1945. The purification and crystallization of rennin. Biochem. J. 39:179.

3. De Baun, R. M., W. M. Connors, and R. A. Sullivan. 1953. The preparation of crystalline rennin and a study of some of its properties. Arch. Biochem. and Biophys. 43:324.

4. Ernstrom, C. A. 1958. Heterogeneity of crystalline rennin. J. Dairy Sci. 41:1663.

5. Kaye, M. A. G., and P. W. Kent. 1953. Ester and lactone linkages in acidic polysaccharides. J. Chem. Soc. :79.

6. Kent, P. W., and M. W. Whitehouse. 1955. Biochemistry of the aminosugars. Academic Press, New York. 311 p.

7. Komarov, S. A. 1935. Isolation of mucoitinsulfuric acid from canine gastric juice. J. Biol. Chem. 109:177.

8. Legg, M. W. 1965. Unpublished data. New Zealand Cooperative Rennet Company, Eltham, New Zealand.

9. Leitch, R. H. 1937. Manufacture of rennet. Congr. intern. tech. et. chim. ind. agr. Compt. rend. 5th Congr. 2:307.

10. Levene, P. A., and F. B. LaForge. 1914. On the conjugated sulphuric acid from tendomucoid. J. Biol. Chem. 18:237.

11. Levene, P. A., and J. Lopez-Suarez. 1918. Mucins and mucoids. J. Biol. Chem. 36:105.

12. Mathews, M. B. 1953. Chondroitinsulfuric acid - a linear polyelectrolyte. Arch. Biochem. and Biophys. 43:181.

13. Meyer, K., and M. M. Rapport. 1951. The mucopolysaccharides of the ground substance of connective tissue. Science 113:596.

14. Morgan, W. T. J., and H. K. King. 1943. The isolation from hog gastric mucin of the polysaccharide-amino acid complex possessing blood group A specificity. Biochem. J. 37:640.

15. New Zealand Department of Agriculture. 1916. Preparation of calves' vells for rennet making. Bull. 61. 2 p.

16. Ostle, B. 1966. Statistics in research. 2nd Ed. The Iowa State University Press, Ames, Iowa. 585 p.

17. Paulson, S., B. Sylven, C. Hirsch, and O. Snellman. 1951. The diffusion rate of various substances in normal bovine "nucleus pulposus." Biochem. Biophys. Acta. 7:207.

18. Pirie, A. 1946. Mucoitin sulphuric acid, chondroitin sulphuric acid, hyaluronic acid and heparin. Biochem. J. 40:xi.

19. Placek, C., V. S. Bavisotto, and E. C. Jadd. 1960. Commercial enzymes by extraction (rennet). Ind. Eng. Chem. 52:2.

20. Smith, H. 1951. Further fractionation of hog gastric mucin; an additional factor in the adjuvant action. Biochem. J. 48:441.

21. Smith, H., and R. C. Gallop. 1953. The "acid polysaccharides" of hog gastric mucosa. Biochem. J. 53:666.

22. Terho, T., K. Hartiala, and I. Hakkinem. 1966. Infrared spectroscopic investigations of a new acid mucopolysaccharide isolated from human gastric juice. Nature 211:198.

23. Van der Burg, B., and A. F. Van der Scheer. 1937. La preparation dune presure parfaitement claire. Congr. intern. tech. et. chim. ind. agr. compt. rend. 5th Congr. 2:321.

24. White, A., P. Handler, and E. L. Smith. 1964. Principles of biochemistry. 3rd Ed. McGraw-Hill Book Company, Inc., New York. 1106 p.

25. Wolfrom, M. L., R. Montgomery, J. V. Karabinos, and P. Rathgeb. 1950. The structure of heparin. J. Amer. Chem. Soc. 72:5796.

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